Staphylococcus aureus products subvert the *Burkholderia cenocepacia*-induced inflammatory response in airway epithelial cells

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Abstract

Introduction. Polymicrobial infections commonly occur within the lower respiratory tract, although there is a paucity of information regarding the host response to multi-species pathogens. Deleterious effects occur frequently as a result of microbe-microbe interactions, although an unexpected protective effect of *Staphylococcus aureus* has been observed in cystic fibrosis patients co-infected with *Pseudomonas aeruginosa* or species belonging to the *Burkholderia cepacia* complex.

Aim. This study aims to investigate the differences in host responses to mono- and co-infection with *S. aureus* and *B. cenocepacia* in 16HBE human airway epithelial cells, interpret the host receptor and signalling events targeted by *S. aureus*, and characterise staphylococcal immunomodulatory factors.

Methodology and Results. The results show that *B. cenocepacia* activates MAPK and NF-κB signalling pathways, subsequently eliciting a robust interleukin (IL)-8 production. However, when airway epithelial cells were co-treated with live *B. cenocepacia* bacteria and *S. aureus* supernatants (conditioned medium), the pro-inflammatory response was attenuated. This anti-inflammatory effect was widely exhibited in *S. aureus* isolates tested and was mediated via reduced MAPK and NF-κB signalling but not via the IL-1 receptor or the tumour necrosis factor receptor modulation. The staphylococcal effectors were characterised as small, heat-stable, non-proteinaceous, and not cell wall-related factors.

Conclusion. This study demonstrates for the first time the host response in a *S. aureus* / *B. cenocepacia* co-infection model and provides insight into a staphylococcal immune evasion mechanism, as well as a therapeutic intervention for excessive inflammation.

Introduction

Lower respiratory tract infections are a major cause of morbidity and mortality globally (Troeger et al., 2017). Individuals with an immune deficiency, chronic obstructive pulmonary disease, or cystic fibrosis (CF) are particularly prone to such respiratory infections (Pragman et al., 2016).

The opportunistic pathogen *Staphylococcus aureus* is one of the major threats that is commonly associated with respiratory infections both in hospital and community environments (Tong et al., 2015). This is not only due to a wide range of virulence factors deployed by *S. aureus* (Haaber et al., 2017; Thammavongsa et al., 2015), but also the rapid emergence of multidrug-resistant strains. *S. aureus* that gains resistance to β-lactam antibiotics as a consequence of adopting the staphylococcal cassette chromosomal *mec* element, becomes methicillin-resistant *S. aureus* (MRSA). This emergence is posing serious challenges to antimicrobial therapy as patients harbouring MRSA are associated with worse clinical prognosis compared to those who never have MRSA (Dasenbrook et al., 2010; Moran et al., 2012).

Burkholderia cepacia complex (Bcc) is a major threat for immunocompromised individuals such as CF patients, as it is transmissible between patients who can develop 'cepacia syndrome' which is a fatal exacerbation of respiratory function as a result of bacteraemia (Hauser and Orsini, 2015; Kenna et al., 2017; LiPuma et al., 1988; Palfreyman et al., 1997). Together with *Pseudomonas aeruginosa* and other species, Bcc are non-fermenting Gram negative bacilli which are not able to utilise glucose in the absence of oxygen. Pathogens of these bacteria have become an increasing challenge in hospital-acquired infections due to the high magnitude of intrinsic antibiotic resistance (Oliveira et al., 2017; Shommu et al., 2015). While the prevalence of *S. aureus* infection is about 60% among CF patients younger than 2

years and peaks between 11 to 17 years old, most Bcc colonisation is acquired after the age of 18 (Cystic Fibrosis Foundation, 2017). Bcc bacteria express a wide range of virulence factors to facilitate the infection process, such as cable pili, flagella, several secretion systems or homologous structures (Leitão et al., 2010; Urban et al., 2005; 2004). In addition, the unusual modification of lipopolysaccharide (LPS) at least partially contributes to the virulence of Bcc. Acylation of lipid A contributes to a much stronger pro-inflammatory response induced by Bcc LPS even compared to LPS from *P. aeruginosa* and *Escherichia coli* (De Soyza et al., 2008). The resulting cytokine storm attracts a large number of immune cells such as neutrophils and mononuclear cells to the lung. If the acute inflammatory response is not resolved, it causes tissue injury which leads to pulmonary fibrosis and possibly develops into a chronic inflammation (Tisoncik et al., 2012).

Chronic pulmonary infection is commonly associated with colonisation with more than one microorganism, but little is known regarding the complicated mechanisms of host-microbe and microbe-microbe interactions and their impact on disease. The microbe-microbe interaction can be synergistic or antagonistic in nature and both forms of interaction can lead to increased antibiotic resistance and virulence (Orazi and O'Toole, 2017; Perez et al., 2014; Ramsey and Whiteley, 2009). Although many studies have reported worse clinical outcomes resulting from microbe-microbe interactions (Bragonzi et al., 2012; Fugère et al., 2014; Korgaonkar et al., 2013), this is not always the case. Contrary to the negative impact by Bcc and *P. aeruginosa*, *S. aureus* co-infection is positively associated with a relatively better lung function, higher survival rate, and delayed lung disease progression (Emerson et al., 2002; Liou et al., 2001; Mayer-Hamblett et al., 2007; Nixon et al., 2001). An *in vivo* study that mimicked the sequential acquisition of early *S. aureus* and late *P. aeruginosa* in the murine lung

shows that pre-infection with *S. aureus* significantly reduced inflammation and the incidence of mortality caused by *P. aeruginosa* but enhanced *P. aeruginosa* persistence (Cigana et al., 2018). We thus hypothesised that the protective effect of *S. aureus* results from dampening the inflammatory response towards Bcc by interfering with the host signalling pathways. Therefore, evaluating whether *S. aureus* colonisation favours the host and understanding the mechanism behind the unexpected protective effect of *S. aureus* may shed light on novel therapies for persistent infections.

In this study, in order to gain insights of the host response to *S. aureus* / *B. cenocepacia* co-colonisation, we assessed the effect of *B. cenocepacia* on the host signalling cascade and inflammatory responses in airway epithelial cells, compared to that of co-infection. Further we identified the host target of staphylococcal immunomodulatory factors, and the nature of immunomodulatory molecules released by *S. aureus*, highlighting the anti-inflammatory modulation of *S. aureus* during Bcc co-infection and potential effects on the clinical outcome.

Material and methods

Bacteria strains and culture

The bacterial strains used in this study are listed in Table 1. Bacteria were cultured in LB broth (Thermo Fisher Scientific) at 37°C with shaking. Bacterial supernatants were collected and filtered through a 0.2 µm membrane. Where indicated, supernatants were heat-treated by incubating at 95°C for 10 minutes. Heat-killed *S. aureus* were prepared by resuspending the overnight culture to the desired multiplicity of infection (MOI) in DMEM (Gibco, Thermo Fisher Scientific), and heating at 100°C for 1 hour.

Proteinase K immobilised on Eupergit® C (Sigma-Aldrich) was used at a concentration of 100 µg/mL in Tris-Cl (50 mM, pH 8) / $CaCl_2$ (10 mM) at 37°C for 1 hour. At the end of digestion, proteinase K was removed by centrifugation at 250 *g* for 10 minutes. Inactivated proteinase K was prepared by heating at 100°C for 1 hour.

Fractionated products of *S. aureus* were prepared by submitting supernatants to 10-kDa- and 3-kDa-cutoff centrifugal concentrators (Vivaspin 20, Sigma-Aldrich), centrifuged at 5000 *g* for 17 minutes and 180 minutes respectively following the manufacturer's instructions. Supernatants were separated into three fractions: >10000 molecular weight cut-off (MWCO), 3000-10000 MWCO, and <3000 MWCO. Filtrates and concentrates were adjusted to the original volume with fresh LB, followed by filter sterilisation.

Human airway epithelial cell culture

Human bronchial epithelial 16HBE (obtained from Prof Dieter C. Gruenert, University of California San Francisco) and human airway epithelial Calu-3 (ATCC) were maintained in DMEM, supplemented with 10% heat-inactivated foetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin (all from Gibco, Thermo Fisher

Scientific). Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. IL-1β and tumour necrosis factor (TNF) α (Peprotech) were used at the concentrations indicated.

Cell viability assay

The cell viability assay with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) assay was used as described before (Wachsmann and Lamprecht, 2012). Briefly, 16HBE cells were plated in 96-well plates (5000 cells/well) and incubated for 24 hours. Next, the medium was replaced by 10% *S. aureus* supernatants for 24 hours. After incubating cells with 12 mM MTT for 2 hours, formazan formed in viable cells was dissolved in DMSO for 10 minutes before the optical density was read at 550 nm.

Detection of Cytokine Production

At the end of stimulation, conditioned medium of 16HBE cells was collected and the concentration of interleukin (IL)-8 was quantified using Maxisorp ELISA plates (Nunc, Thermo Fisher Scientific) and a commercially available ELISA kit (eBioscience, Thermo Fisher Scientific) according to the manufacturer's instructions.

Western Blot Analysis

Following stimulation, cell lysates were prepared and the concentration of total protein was quantified using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Protein samples were resolved on an SDS-PAGE gel, and immunoblotted on a nitrocellulose membrane (Bio-Rad). The membrane was probed with antibodies anti phospho-NF-κB-p65, phospho-p38, phospho-Erk, and p38 (Cell Signaling Technology), followed by incubation with secondary antibodies (Li-Cor Biosciences).

Quantitative analysis of the signal was performed using the Li-Cor Odyssey Clx imaging system.

Statistical Analysis

Data were expressed as the mean ± standard error of the mean (SEM) for n=3 independent biological repeats. Results were compared using Graphpad Prism and one-way or two-way ANOVA, followed by Tukey or Dunnett's multiple comparisons test, with $p<0.05$ considered significant.

Results

B. cenocepacia-stimulated IL-8 response is suppressed by *S. aureus* products.

IL-8 plays an important role at the early stage of infection by chemoattracting granulocytes such as neutrophils (Perret et al., 2012). Hence, the level of IL-8 is a biomarker of pro-inflammatory responses and inflammation (Dennehy et al., 2017; Palfreyman et al., 1997). *B. cenocepacia* induced a robust increase in IL-8 response in 16HBE cells (Figure 1A). Although supernatants (in LB medium) of *S. aureus* MRSA252 or MSSA NCTC 6571 did not change IL-8 production compared to the control, they significantly downregulated IL-8 induced by *B. cenocepacia* (23% and 31% less than the *B. cenocepacia*-treated group, respectively). In contrast, heat-killed *S. aureus* did not have the suppressing effect (Figure 1B).

The same experiments were done with a collection of *S. aureus* isolates, either supernatants (Figure 1C). Supernatants from all the tested isolates downregulated the level of IL-8 production by different amounts (5.4%-38.0%). Although some of the differences were small, consistent changes were seen within each experiment and these were statistically significant ($p<0.05$). The ability of heat-killed bacteria to inhibit *B. cenocepacia*-elicited IL-8 production (Figure 1D). However, whole heat-killed bacteria had no effect on *B. cenocepacia*-induced IL-8 production. These data indicate that anti-inflammatory activity in these experiments is expressed in secreted products but not heat-stable *S. aureus* cell surface structures. This contrasts with the nasal isolate *S. aureus* anti-inflammatory activity reported by Peres et al. (2015), which was exhibited by whole bacteria subjected to the same heat treatment conditions.

To confirm the suppressing effect is not due to cell damage, an MTT assay was used to assess cell viability (Figure 1E). For the majority of *S. aureus* strains, their

supernatants did not cause epithelial cell damage, indicating that there was no causality between IL-8 suppression and induction of epithelial cell damage. Exceptions were SH1000 and RN4220 which did reduce cell viability and, interestingly, these alone caused a doubling in IL-8 production (Figure 1C).

MSSA NCTC 6571 products block MAPK p38 and Erk, and NF-κB signalling activated by *B. cenocepacia*

The recognition of conserved microbial structures of pathogens by pattern recognition receptors leads to activation of MAPK and NF-κB signalling pathways, subsequently initiating the expression of pro-inflammatory genes to mount an immune response (Pandey et al., 2014). NF-κB and MAPK pathways contribute to IL-8 expression at multiple levels (Hoffmann et al., 2002). Since *B. cenocepacia* induced a strong IL-8 production, the effects of *B. cenocepacia* on MAPK and NF-κB signalling in 16HBE cells were assessed. Cells were challenged with *B. cenocepacia* at an MOI of 5 from 5 minutes to 180 minutes (Figure 2 A-D). For the MAPK family, phosphorylation of p38 was maximal at 120 minutes (200% more than time 0), while phosphorylation of Erk peaked at 5 minutes (156% more than time 0), followed by a decrease and then a secondary rise at 120 minutes (133% more than time 0). For NF-κB signalling, *B. cenocepacia* upregulated phosphorylation of p65, particularly after 120 minutes.

In order to study the modulation on host signalling by this co-stimulation model, 16HBE cells were treated with *B. cenocepacia* (5 MOI) and 10% *S. aureus* supernatant for 2 hours (Figure 2E-H). *B. cenocepacia* led to phosphorylation of p38, Erk, and p65, whereas *S. aureus* supernatant alone did not change any tested signalling events. Notably, MSSA NCTC 6571 but not MRSA252 supernatant reduced phosphorylation of p38, Erk and p65 stimulated by *B. cenocepacia.* The reduction in p38 and p65 signalling was significant compared with *B. cenocepacia* alone ($p<0.05$). This strain

selectivity is consistent with the differential inhibition of the pro-inflammatory response (Figure 1A) by MSSA NTCT 6571 and MRSA252. Therefore, these findings suggest that MSSA NCTC 6571 supernatant may suppress the pro-inflammatory effect of *B. cenocepacia* by inhibiting MAPK and NF-κB signalling.

Characterisation of anti-inflammatory factors in *S. aureus* supernatant

To investigate the nature of anti-inflammatory components, *S. aureus* supernatants were heated at 95°C for 10 minutes. These heat-treated supernatants were still able to inhibit the *B. cenocepacia*-stimulated IL-8 response (Figure 3A), indicating that the components are heat stable.

Further, *S. aureus* supernatants were treated with solid-phase proteinase K for 1 hour at 37°C before removal to avoid any effect of the proteinase on released IL-8. Proteinase K-digested supernatants did not restore IL-8 secretion caused by *B. cenocepacia*; rather, IL-8 secretion was nearly reduced to the baseline (Figure 3B). Further, the 16HBE cultures incubated with proteinase-treated supernatant alone did not inhibit basal IL-8 levels (Figure 3B), indicating that no amounts of protease K was carried over into the production assay. Heat-inactivated proteinase K-treated supernatants displayed similar inhibition level as untreated supernatants (Figure 1A), indicating the components are either non-proteinaceous or proteinase K-resistant.

To assess the approximate molecular weight of the secretory *S. aureus* components causing a reduction in *B. cenocepacia*-induced IL-8 production, *S. aureus* supernatants were separated into different molecular cut-off fractions using selective membranes (Figure 3C). Components in the supernatants with a molecular weight less than 3 kDa, but not above 3 kDa reduced IL-8 production back to the same level as the control, which indicates the anti-inflammatory factors are small molecules.

As staphylococcal virulence factors (such as SpA and Panton-Valentine Leucocidin) target the IL-1 receptor (IL-1R) and tumour necrosis factor receptor (TNFR), leading to IL-8 expression (Gomez et al., 2004; Labrousse et al., 2014), in order to elucidate whether the *S. aureus*-induced anti-inflammatory response was selective for bacterial stimuli, 16HBE cells were treated with IL-1β and TNFα, resulting in the same amount of IL-8 as the *B. cenocepacia*-treated group (Figure 3D). However, contrary to the reduction seen with *S. aureus* supernatant and J2315 co-stimulation, *S. aureus* supernatants did not suppress IL-1β / TNFα-induced IL-8 production, indicating that IL-1R or TNFR signalling is not associated with this process and the suppressive effect appears to be targeted toward pathways involved in the *B cenocepacia* IL-8 response.

Discussion

This study is the first to demonstrate the host response caused by *S. aureus* / *B. cenocepacia* co-infection in airway epithelial cells. We demonstrate that *S. aureus* secreted products, but not cell wall-related components are able to suppress the pro-inflammatory response induced by *B. cenocepacia*. The active compound(s) are primarily characterised as small, heat-stable, non-proteinaceous, and targeting MAPK and NF-κB signalling.

Previous studies have concentrated on the interactions between *S. aureus* and *P. aeruginosa* as they are two prevalent pathogens involved in CF lung infections (Ahlgren et al., 2015; Baldan et al., 2014; Chekabab et al., 2015; DeLeon et al., 2014; Fugère et al., 2014; Orazi and O'Toole, 2017). However, the impact of *S. aureus* / Bcc interactions on host responses has never been assessed. Herein, we demonstrate that in the context of *S. aureus* / *B. cenocepacia* co-infection, supernatants from *S. aureus* isolates subverted *B. cenocepacia* live bacteria-induced IL-8 production in airway epithelial cells, suggesting that *S. aureus* releases anti-inflammatory compounds into the extracellular milieu. This effect of *S. aureus* provides a possible explanation of the protective effect of *S. aureus* colonisation on lung function and patient survival (Liou et al., 2001; Mayer-Hamblett et al., 2007).

Interestingly, all the supernatants from *S. aureus* isolates tested were able to subvert *B. cenocepacia*-induced IL-8 production ($p<0.05$). Even hyperinflammatory isolates such as SH1000 and RN4220, where the supernatants induced strong IL-8 production (Figure 1C), were able to reduce the inflammatory response, indicating the components may be widely expressed in *S. aureus*. The secretome of *S. aureus* involves a large arsenal of immune evasion factors that inhibit complement, chemotaxis, phagocytosis, and induce neutrophil lysis. A whole genome study divided

88 *S. aureus* isolates into 25 clonal complex lineages and investigated 43 immune evasion genes (McCarthy and Lindsay, 2013). The results revealed that although genes encoding leucocidin are highly conserved, there are variants among other genes, indicating each lineage applies different strategies and functions of immune evasion.

The MAPK family and NF-κB signalling pathways are known to play key roles in the host response to bacterial pathogens by regulating the expression of cytokines and chemokines (Krachler et al., 2011). However, pathogens have evolved to exploit these pathways. For example, *B. cenocepacia* K56-2 can significantly upregulate phosphorylation of Akt, Erk, and NF-κB in 16HBE cells within 30 minutes of stimulation (Gillette et al., 2013). In parallel, *B. cenocepacia* BC7 activates phosphorylation of p38 and JNK in CF bronchial epithelial IB3 cells, whereas these pathways remain unphosphorylated in contact with an environmental *B. cepacia* strain (Sajjan et al., 2008). As K56-2 and BC7 both belong to the highly virulent ET-12 lineage, this indicates that hyperactivation of host signalling is related to more severe lung inflammation caused by *B. cenocepacia* isolates.

In our model, *B. cenocepacia* caused phosphorylation of p38, Erk, and p65 at 120 minutes post-infection. Of note, after a transient increase at 5 minutes post-infection, phosphorylation of Erk peaked again at 120 minutes. The similar bi-phasic activation pattern of Erk and p38 was observed in the response to *B. pseudomallei* both *in vitro* and *in vivo* (D'Elia et al., 2017). Thirty-minutes post-infection, murine alveolar macrophages exhibited transient phosphorylation of p38 and Erk1, and at 2 h post-infection exhibited sustained activation. As Erk regulates apoptosis in response to oxidative stress (Li et al., 2016), D'Elia et al. (2017) proposed that the transient activation at the early stage is associated with host defence, while during the latter

stage of infection host cells might initiate apoptosis as indicated by continuous Erk activation, and thereby *B. pseudomallei* gains time for intracellular proliferation by delaying apoptosis through modulate the dynamics of Erk activation.

Upon co-stimulation with *B. cenocepacia* live bacteria and *S. aureus* supernatant, activation of MAPK and NF-κB was assessed where MSSA NCTC 6571 supernatant abolished phosphorylation of p38, Erk, and p65 caused by *B. cenocepacia*. MRSA252 supernatant did not affect either baseline or *B. cenocepacia*-induced phosphorylation, which correlates with its lower inhibitory ability on IL-8 production compared to MSSA NCTC 6571. Our results are in partial agreement with the results displayed by Chekabab et al., (2015) as they showed *S. aureus* supernatants inhibited *P. aeruginosa* supernatant-induced inflammatory response through blocking NF-κB activation but not p38. However, Chekabab et al. (2015) stimulated airway epithelial Beas-2B cells with synthetic Toll-like receptor (TLR)-2/1 agonist Pam_3CSK4 but not *P. aeruginosa*, but bacteria themselves contain far more complicated ligands which could potentially activate the MAPK family. Additionally, the downstream signalling cascades engaged in the anti-inflammatory response may be cell type-specific. For example, in human peripheral blood mononuclear cells, among a group of nasal *S. aureus* isolates, some isolates induce anti-inflammatory IL-10 production through PI3K/Akt/mTOR and Erk signalling whereas some induce pro-inflammatory TNFα through a p38-dependent pathway (Peres et al., 2015). This suggests that *S. aureus* manipulates different signalling cascades to initiate immune tolerance through heterogenicity expression of ligands in the process of infection.

In further experiments, the treatments of heat, proteinase K, and different molecular weight cut-off fractions were examined in order to determine the nature of the anti-inflammatory compounds in *S. aureus* supernatants. Consistent with previous studies,

the compounds were characterised as small (<3 kDa) and heat-resistant (Chekabab et al., 2015; Tajima et al., 2006). As secondary or tertiary structures of proteins are usually disrupted during boiling, the staphylococcal factors are unlikely to be proteins. Similarly, when co-stimulated with *B. cenocepacia*, IL-8 production elicited by proteinase K-treated *S. aureus* supernatants showed a 50% decrease compared to deactivated proteinase K-treated *S. aureus* supernatants, indicating that anti-inflammatory components are less likely to be peptides due to the nonspecific protease activity of proteinase K. A possible explanation is that proteinaceous pro-inflammatory components in *S. aureus* supernatants were degraded by proteinase K and thus the anti-inflammatory effectors overpowered the pro-inflammatory counterparts. For example, the major virulence factor SpA not only contributes to immune evasion by binding to immunoglobulin, but also elicits a strong pro-inflammatory response through interacting with tumour necrosis factor receptor 1 and epidermal growth factor receptor (Gomez et al., 2004; Graille et al., 2000; Soong et al., 2011). So far only one research group reported small, heat-, and protease-resistant molecules derived from MRSA and *S. epidermidis* conditioned medium that induce nitric oxide in human upper airway epithelial cells (Carey et al., 2016; 2015). Although these studies did not assess production of cytokines and chemokines in response to the factors, the result strongly indicates the existence of small, heat- and protease-resistant molecules that are secreted by *S. aureus*.

We also investigated whether *S. aureus* factors interact with other receptors such as IL-1R and TNFR that promote the inflammatory response. The results showed that the inhibitory effect of *S. aureus* secreted products was *B. cenocepacia* stimuli-specific, as *S. aureus* supernatants had no effect on IL-1β / TNFα-induced IL-8 production. This is not surprising as *S. aureus* can colonise human airway asymptomatically, and

therefore is able to trigger a low level of inflammatory response and even suppress it in order to induce immune tolerance. Known staphylococcal factors that antagonise the vital microbial sensor TLRs, include staphylococcal superantigen-like protein 3 and TIR domain protein targeting TLR2 (Askarian et al., 2014; Koymans et al., 2017), and phenol-soluble modulin α1-3 targeting TLR4 (Chu et al., 2018). However, little is known about whether the immunosuppression of *S. aureus* is beneficial to the host. To further explore our findings, the impact of *S. aureus* on *B. cenocepacia* infection need to be investigated using an *in vivo* respiratory infection model. Our findings for the first time illustrate the host response to *S. aureus* and *B. cenocepacia* co-infection in the airway epithelial cells, and reveal staphylococcal anti-inflammatory factors that suppress *B. cenocepacia*-induced IL-8 production through blocking MAPK and NF-κB signalling. To conclude, we reveal immune evasion factors secreted by *S. aureus* that are small, heat-stable, and non-proteinaceous, which reduce the pro-inflammatory response induced by CF-isolated *B. cenocepacia* in 16HBE cells. These results suggest a potential treatment for patients with chronic pulmonary infections, whereby drugs targeting MAPK and NF-κB pathways could be applied to reduce inflammation without facilitating infections.

Author statement

The authors declare that there are no conflicts of interest.

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649 **Table 1 Bacteria strains**

Strain	Characteristics	Reference
MRSA252	Hospital-acquired MRSA, fatal post-op septicemia	Holden et al., 2004
MSSA NCTC 6571	Methicillin-sensitive *S. aureus*	
MSSA Newman	Isolated from a secondary osteomyelitis infection of a TB patient	Baba et al., 2008
MSSA209		Collins et al., 2010
MRSA41		Collins et al., 2010
MSSA SH1000	Laboratory strain, derivative of NCTC 8325 by complementation of *rsbU*	O'Neill, 2010
MSSA RN4220	Derivative of NCTC8325-4, with *agrA* mutation	Nair et al., 2011
MRSA USA400	Community-associated *S. aureus*	Voyich et al., 2006
B. cenocepacia J2315	Epidemic CF-related ET12 lineage	Holden et al., 2009

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Figure 1 *S. aureus* supernatant has anti-inflammatory on *B. cenocepacia*-induced IL-8 response.

IL-8 production in 16HBE cells treated with *B. cenocepacia* (5 MOI), in the presence or absence of **(A)** 10% *S. aureus* supernatants, or **(B)** heat-killed *S. aureus* (5 MOI) for 24 hours. The experiments were validated using **(C)** 10% supernatants from *S. aureus* isolates or **(D)** heat-killed *S. aureus* and *B. cenocepacia* (MOI 5) co-stimulation for 24 hours. In **(A)** and **(C),** 10% LB was used as a control. Data represent mean ± SEM of duplicate samples from three identical experiments. Significance was compared to *B. cenocepacia*-treated cells using 2-way ANOVA followed by Dunnett's multiple comparison test (*, $p<0.05$).

(E) Cytotoxicity of *S. aureus* supernatants on 16HBE cells was assessed using MTT assay. The control was treated with 10% LB. Results are presented as a percent of the control, mean ± SEM from three independent experiments. Significance was compared to the control using 1-way ANOVA followed by Dunnett's multiple comparison test.

Figure 2 Effect of *S. aureus* supernatants on by *B. cenocepacia*-activated MAPK and NF-κB signalling.

16HBE cells were infected by *B. cenocepacia* unwashed culture at an MOI of 5 for the indicated time. TNFα (20 ng/mL) for 5 minutes was used as the positive control. **(A)** Western blot analysis for levels of phosphorylated p38, phosphorylated Erk, phosphorylated p65, and p38 in cell lysate followed by *B. cenocepacia* stimulation. Phosphorylation of p38 **(B)**, ERK **(C)**, p65 **(D)** of cell lysates were measured by western blot and normalised against total p38.

16HBE cells were treated with 10% LB as the control, *B. cenocepacia* (5 MOI), with the presence or absence of 10% *S. aureus* supernatant for 2 hours. **(E)** Western blot analysis for levels of phosphorylated p38, phosphorylated Erk, phosphorylated p65, and p38 in cell lysate following *S. aureus* supernatants and *B. cenocepacia* co-stimulation. Phosphorylation of p38

(F), Erk **(G)** and p65 **(H)** of cell lysates were measured using western blot and normalised against total p38.

Protein bands representative of three independent experiments. Data are shown as mean ± SEM. *, $p<0.05$ significant reduction compared to *B. cenocepacia*-treated group using 2-way ANOVA followed by a one-tailed Dunnett's multiple comparisons test.

Figure 3 Characterisation of anti-inflammatory components in *S. aureus* supernatants.

16HBE cells were treated with *B. cenocepacia* (5 MOI), in the presence or absence of 10% **(A)** heat-treated supernatants, **(B)** proteinase K (PK)-treated *S. aureus* supernatants, **(C)** *S. aureus* supernatant fractions containing metabolites of >10 kDa, >3 kDa, and <3 kDa for 24 hours. IL-8 production was analysed using ELISA. Data represent mean ± SEM from 3 independent experiments performed in duplicate. *, $p<0.05$ significance compared to *B. cenocepacia*-treated group using 2-way ANOVA followed by Dunnett's multiple comparisons test.

(D) 16HBE cells were co-treated with IL-1 (1 ng/mL) / TNFα (10 ng/mL) and 10% *S. aureus* supernatants for 24 hours. *B. cenocepacia* (5 MOI) was the positive control. Data represent mean ± SEM from 3 independent experiments performed in duplicate. *, $p<0.05$ compared to IL-1β / TNFα-treated group or with IL-1/ TNFα -treated group using 2-way ANOVA followed by Tukey's multiple comparisons test.

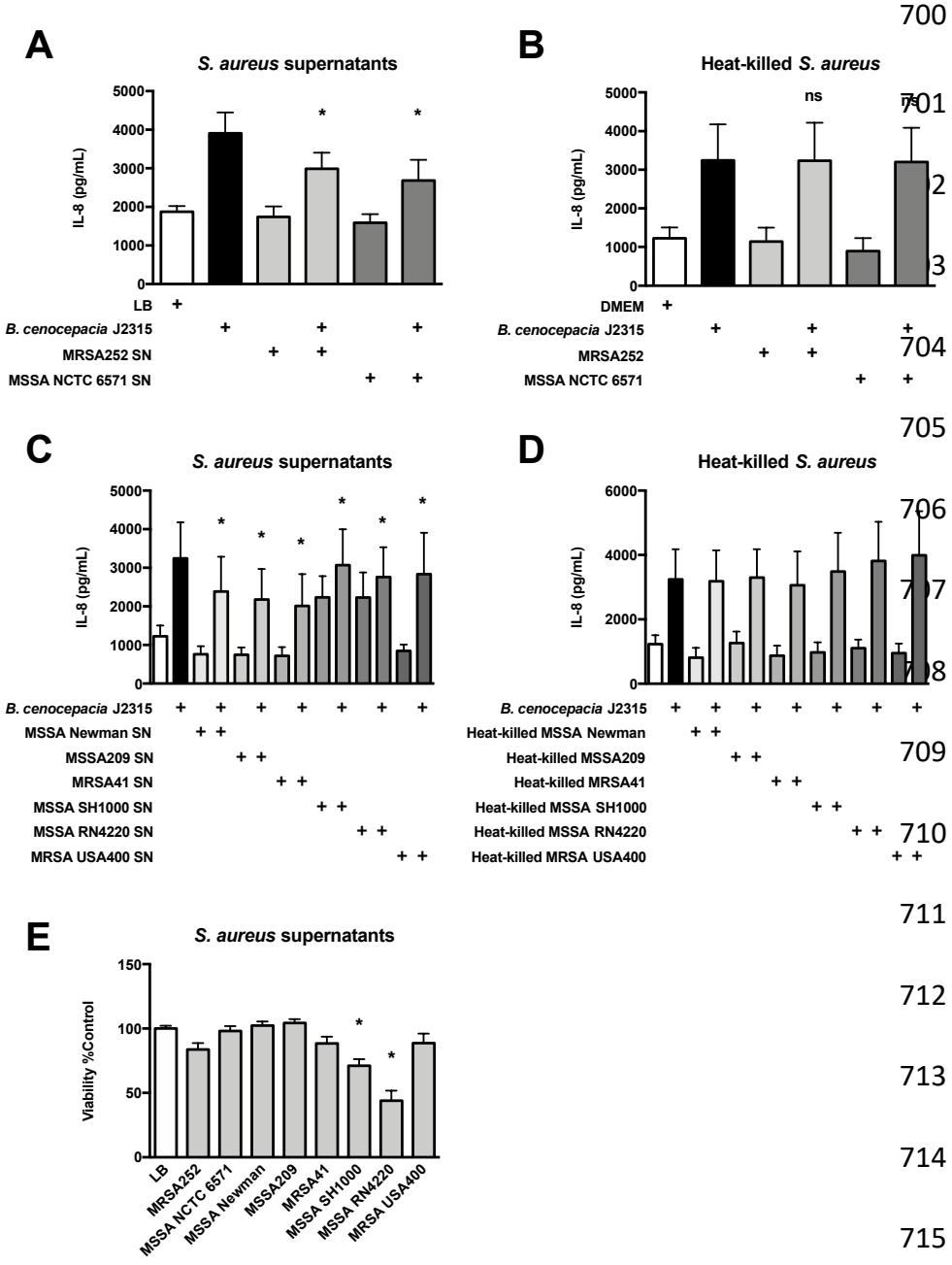
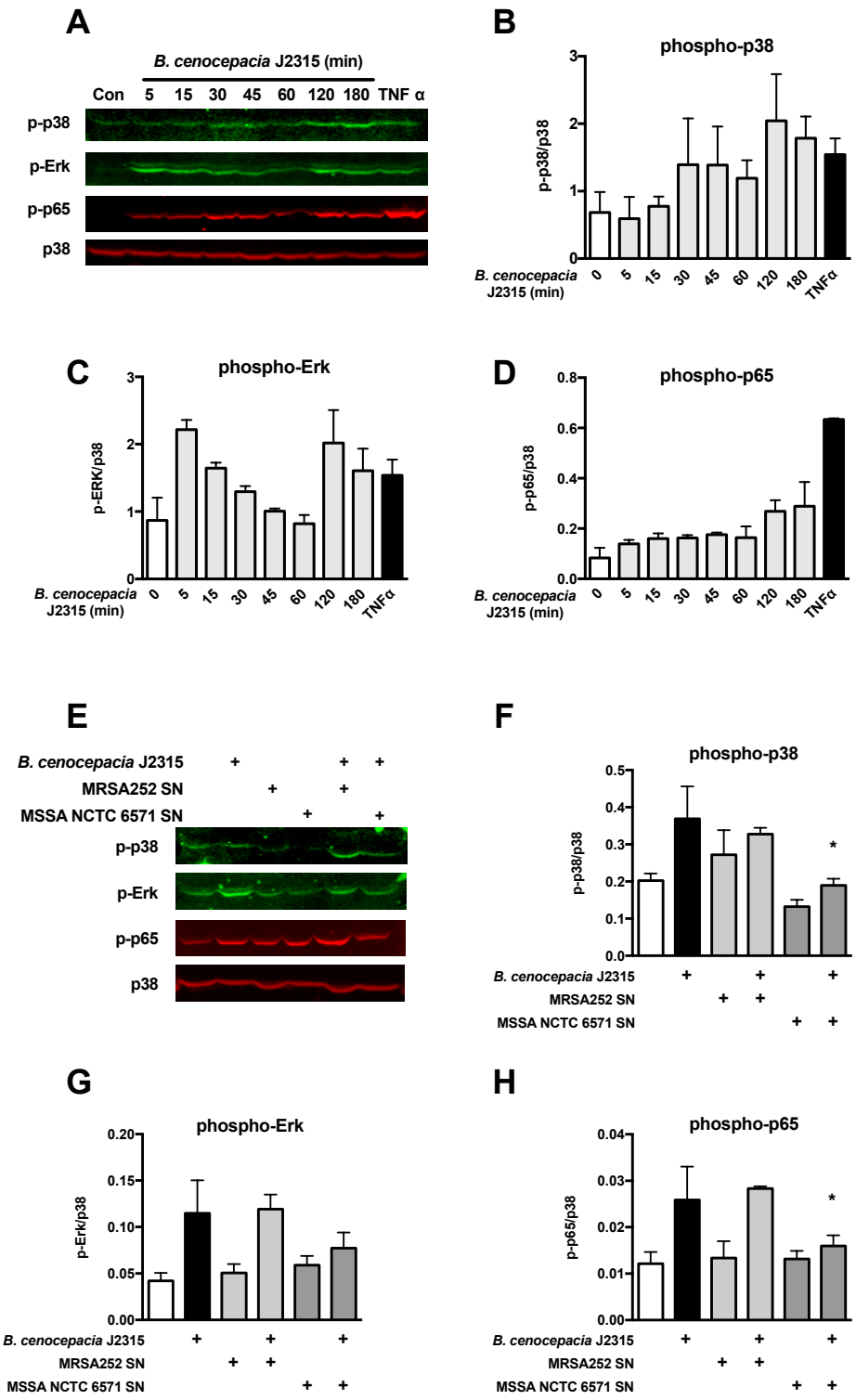

A
S. aureus supernatants
IL-8 (pg/mL)
LB
B. cenocepacia J2315
MRSA252 SN
MSSA NCTC 6571 SN
B
Heat-killed S. aureus
ns
DMEM
MRSA252
MSSA NCTC 6571
C
S. aureus supernatants
MSSA Newman SN
MSSA209 SN
MRSA41 SN
MSSA SH1000 SN
MSSA RN4220 SN
MRSA USA400 SN
D
Heat-killed S. aureus
Heat-killed MSSA Newman
Heat-killed MSSA209
Heat-killed MRSA41
Heat-killed MSSA SH1000
Heat-killed MSSA RN4220
Heat-killed MRSA USA400
E
S. aureus supernatants
Viability %Control
LB
MRSA252
MSSA NCTC 6571
MSSA Newman
MSSA209
MRSA41
MSSA SH1000
MSSA RN4220
MRSA USA400

Figure 1



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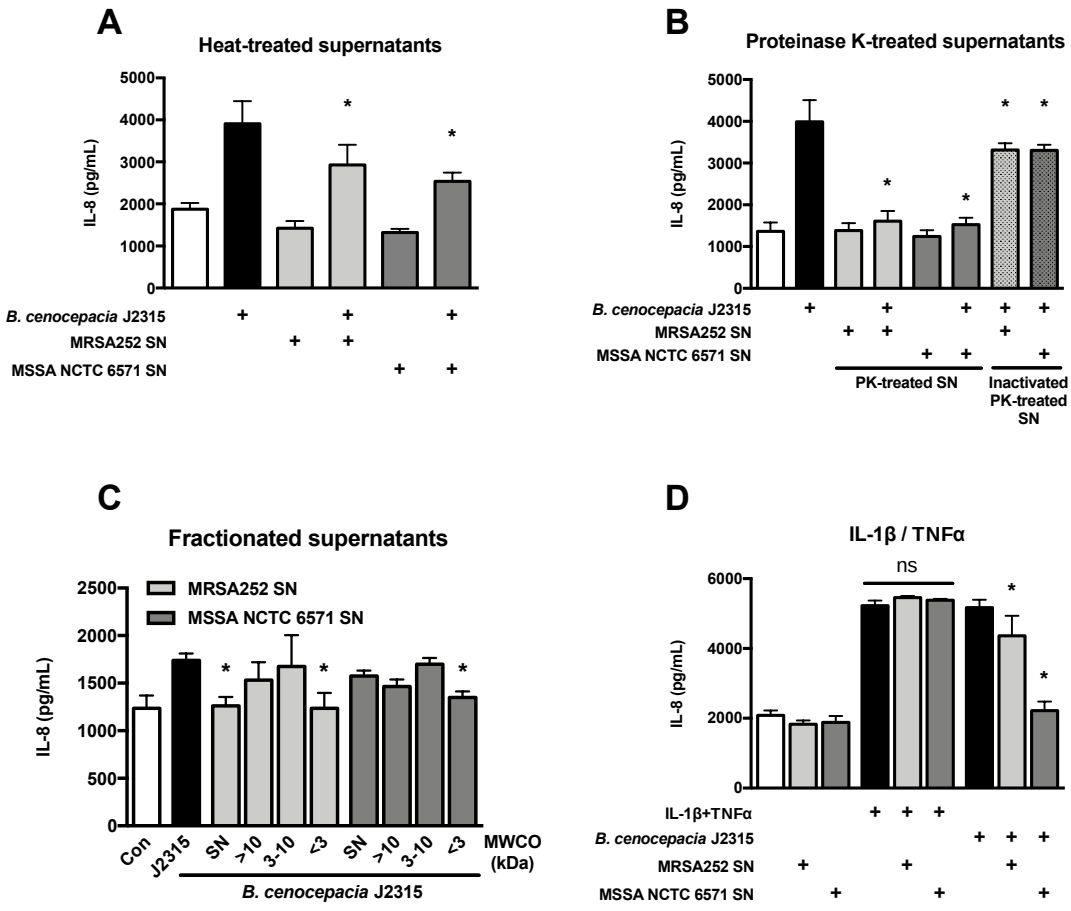
721 Figure 2



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723 Figure 3